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| <b>(51) International Patent Classification <sup>6</sup> :</b><br><b>G01N 33/574, C12Q 1/68</b>   | <b>A1</b> | <b>(11) International Publication Number:</b> <b>WO 97/48982</b><br><b>(43) International Publication Date:</b> 24 December 1997 (24.12.97)  |
| <b>(21) International Application Number:</b> PCT/US97/10306<br><b>(22) International Filing Date:</b> 13 June 1997 (13.06.97)<br><br><b>(30) Priority Data:</b><br>08/664,857      17 June 1996 (17.06.96)      US<br><br><b>(71) Applicant:</b> CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 300 Longwood Avenue, Boston, MA 02115 (US).<br><br><b>(72) Inventors:</b> ZETTER, Bruce, R.; 120 Eliot Avenue, West Newton, MA 02165 (US). BAO, Lere; 1878 Beacon Street #3, Brookline, MA 02146 (US).<br><br><b>(74) Agents:</b> RESNICK, David, S. et al.; Dike, Bronstein, Roberts & Cushman, LLP, 130 Water Street, Boston, MA 02109 (US).   |           | <b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).<br><br><b>Published</b><br><i>With international search report.<br/>         Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| <b>(54) Title:</b> A METHOD FOR DIAGNOSIS AND PROGNOSIS OF CANCER<br><br><b>(57) Abstract</b><br><p>The present inventors have discovered that humans have a gene that encodes a novel protein of the thymosin <math>\beta</math> family. This novel protein, herein referred to as thymosin <math>\beta</math> 15, has the ability to bind and sequester G-actin, like other members of the thymosin <math>\beta</math> family, but unlike what is known about other members also directly regulates cell motility in prostatic carcinoma cells. A cDNA of the human thymosin <math>\beta</math> 15 gene (SEQ ID NO: 1) and having deduced the amino acid sequence (SEQ ID NO: 2) was isolated. The present inventors have shown that enhanced transcripts (mRNA) and expression of the thymosin <math>\beta</math> 15 gene in non-testicular cells has a high correlation to disease state in a number of cancers, such as prostate, lung, melanoma and breast cancer, particularly metastatic cancers. Accordingly, discovering enhanced levels of transcript or gene product in non-testicular tissues can be used in not only a diagnostic manner, but a prognostic manner for particular cancers.</p> |           |  |

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## Background of the Invention

While different forms of cancer have different properties, one factor which many cancers share is that, in order to be fatal, they must metastasize. Until such time as metastasis occurs, a tumor, although it may be malignant, is confined to one area of the body. This may cause discomfort and/or pain, or even lead to more serious problems, but if it can be located, it may be surgically removed and, if

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done with adequate care, cause no further problems. However, once metastasis sets in, cancerous cells have invaded the body and while surgical resection may remove the parent tumor, this does not address other tumors. Only chemotherapy, or some particular form of targeting therapy, then stands any chance of success.

The process of tumor metastasis is a multistage event involving local invasion and destruction of intercellular matrix, intravasation into blood vessels, lymphatics or other channels of transport, survival in the circulation, extravasation out of the vessels in the secondary site and growth in the new location (Fidler, et al., *Adv. Cancer Res.* 28, 149-250 (1978), Liotta, et al., *Cancer Treatment Res.* 40, 223-238 (1988), Nicolson, *Biochim. Biophys. Acta* 948, 175-224 (1988) and Zetter, *N. Eng. J. Med.* 322, 605-612 (1990)). Success in establishing metastatic deposits requires tumor cells to be able to accomplish these steps sequentially. Common to many steps of the metastatic process is a requirement for motility. The enhanced movement of malignant tumor cells is a major contributor to the progression of the disease toward metastasis. Increased cell motility has been associated with enhanced metastatic potential in animal as well as human tumors (Hosaka, et al., *Gann* 69, 273-276 (1978) and Haemmerlin, et al., *Int. J. Cancer* 27, 603-610 (1981)).

Tumor angiogenesis is essential for both primary tumor expansion and metastatic tumor spread, and angiogenesis itself requires ECM degradation (Blood et al., *Biochim. Biophys. Acta* 1032:89-118 (1990)). Thus, malignancy is a systemic disease in which interactions between the neoplastic cells and their environment play a crucial role during evolution of the pathological process (Fidler, I. J.,

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*Cancer Metastasis Rev.* 5:29-49 (1986)).

Identifying the alterations in gene expression which are associated with malignant tumors, including those involved in tumor progression, is clearly a prerequisite not only for a full understanding of cancer, but also to develop new rational therapies against cancer.

A further problem arises, in that the genes characteristic of cancerous cells are very often host genes being abnormally expressed. It is quite often the case that a particular protein marker for a given cancer while expressed in high levels in connection with that cancer is also expressed elsewhere throughout the body, albeit at reduced levels.

Prostatic carcinoma is the most prevalent form of cancer in males and the second leading cause of cancer death among older males (Boring, et al., *Cancer J. Clinicians*, 7-26 (1994)). Clinically, radical prostatectomy offers a patient with locally contained disease an excellent chance for cure. Unfortunately, if diagnosed after metastases are established, prostatic cancer is a fatal disease for which there is no effective treatment to significantly increase survival. Recent advances in prostatic cancer diagnosis has allowed the earlier detection of human prostate cancer by use of the PSA test (Catalona, et al., *J. Urol.*, 151, 1283-1290 (1994)). Unfortunately, this early detection has not been accompanied by an improvement in determining which tumors may progress to the metastatic stage (Cookson, et al., *J. Urology* 154, 1070-1073 (1995) and Aspinall, et al., *J. Urology* 154, 622-628 (1995)). Since many individuals having prostate cancer are not adversely effected by the cancer, considerable controversy has arisen as to the use of such tests. Thus, methods for early detection and

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early appreciation of the potential for or of the severity of the cancer, that can be taken into account in treatment of, for example, metastatic disease are desirable.

5            Bao and Zetter reported in an abstract presented at the American Association for Cancer Research annual meeting (March 18-22, 1995) the differential expression of a novel mRNA expressed in high-metastatic rat tumor cell lines, but not in a low metastatic variant. cDNA was isolated and was reported to encode a protein with 68%  
10        identity to the rat thymosin  $\beta$ 4. However, the nucleotide sequence and the deduced amino acid sequence were not reported.

#### Summary of the Invention

          We have now discovered that humans have a gene that encodes  
15        a novel protein of the thymosin  $\beta$  family. This novel protein, herein referred to as thymosin  $\beta$ 15, has the ability to bind and sequester G-actin, like other members of the thymosin  $\beta$  family, but unlike what is known about other members also directly regulates cell motility in prostatic carcinoma cells. We have isolated a cDNA of the human  
20        thymosin  $\beta$ 15 gene (SEQ ID NO: 1) and have deduced the amino acid sequence (SEQ ID NO: 2). We have shown that enhanced transcripts (mRNA) and expression of the thymosin  $\beta$ 15 gene in non-testicular cells has a high correlation to disease state in a number of cancers, such as prostate, lung, melanoma and breast cancer, particularly metastatic  
25        cancers. Accordingly, discovering enhanced levels of transcript or gene product in non-testicular tissues can be used in not only a diagnostic manner, but a prognostic manner for particular cancers.

          In a first aspect the present invention provides a method of

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diagnosing cancer, especially prostate, lung, melanoma and breast cancer, in a patient by measuring levels of thymosin  $\beta$ 15 in a biological specimen obtained from the patient. Levels of thymosin  $\beta$ 15 in the sample greater than a base line level is indicative of cancer. Biological  
5 specimens include, for example, blood, tissue, serum, stool, urine, sputum, cerebrospinal fluid and supernatant from cell lysate. Preferably, one uses tissue specimens. The determination of base lines and comparison levels is by standard modes of analysis based upon the present disclosure.

10

In another aspect, the present invention provides a method of prognosis in an individual having cancer, especially prostate, lung, melanoma and breast cancer, by measuring levels of thymosin  $\beta$ 15 in a tumor sample obtained from a patient to be tested. Expression of  
15 thymosin  $\beta$ 15 in said tumor sample greater than a base line level for that particular tissue indicates a higher risk of tumor metastasis.

In yet another aspect, the present invention provides a method for determining the metastatic potential of a tumor by measuring the  
20 level of thymosin  $\beta$ 15 expression in the tumor. Expression of thymosin  $\beta$ 15 in said tumor greater than a base line level for that particular tissue indicates an increased metastatic potential.

In yet another embodiment, changes in condition can be  
25 monitored by comparing changes in thymosin  $\beta$ 15 expression levels in the tumor in that subject over time.

In the methods of the present invention, levels of thymosin  $\beta$ 15 can be ascertained by measuring the protein directly or indirectly by

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measuring transcript (mRNA) encoding thymosin  $\beta$ 15. mRNA levels can be measured, for example, using an RNA dependent polymerase chain reaction, e.g., reverse transcriptase PCR, or Northern blot analysis.

5

Base line levels can readily be determined by measuring levels of thymosin  $\beta$ 15 in sample of disease free individuals.

The present invention also provides of a method for measuring  
10 human thymosin  $\beta$ 15 levels using an antibody or antibody fragment which selectively binds human thymosin  $\beta$ 15. This method comprises the steps of:

- a. contacting a sample or preparation thereof with an antibody or antibody fragment which selectively binds human thymosin  $\beta$ 15, and
- 15 b. detecting whether said antibody or said antibody fragment is bound by said sample and thereby measuring the levels of human thymosin  $\beta$ 15 present, wherein a negative result is indicative of a better prognosis than a positive result.

20 The term "unique fragment" refers to a portion of the nucleotide sequence or polypeptide of the invention that will contain sequences (either nucleotides or amino acid residues) present in thymosin  $\beta$ 15 (SEQ ID NO: 2) but not in other member of the thymosin family. This can be determined when the hybridization profile of that fragment  
25 under stringent conditions is such that it does not hybridize to other members of the thymosin family. Such fragments can be ascertained from Figure 2. A preferred set of unique fragments are those that contain, or contain polynucleotides that encode, amino acid 7 to 12 of SEQ ID NO: 2, amino acid 21 to 24 of SEQ ID NO: 2 and amino acid



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36 to 45 of SEQ ID NO: 2. Preferably, the unique nucleotide sequence fragment is 10 to 60 nucleotides in length, more preferably, 20 to 50 nucleotides, most preferably, 30 to 50 nucleotides. Preferably, the unique polypeptide sequence fragment is 4 to 20 amino acids in length,  
5 more preferably, 6 to 15 amino acids, most preferably, 6 to 10 amino acids.

### Description of the Drawings

Figure 1 is the nucleotide sequence (SEQ ID NO.: 1) of T $\beta$ 15  
10 cDNA and the predicted amino acid sequence (SEQ ID NO.: 2) (single-letter code). The sequence numbers of the nucleotides and amino acids are indicated on the right side of the sequences. The translation initiation codon ATG is underlined, and the termination codon TAA is marked with an asterisk. A putative actin binding region  
15 is underlined. These sequence data are available from GenBank under accession number U25684.

Figure 2 shows the alignment of the deduced T $\beta$ 15 protein sequence and some of the other  $\beta$  thymosin isoforms. Regions of  
20 amino acid identity are represented by white letters boxed in black. Unboxed black letters correspond to nonidentical regions. Dots correspond to gaps introduced in the sequence to optimize alignment.

Figures 3A and 3B show in situ hybridization with antisense  
25 riboprobe for T $\beta$ 15 on prostatic adenocarcinoma patients. Figure 3A shows differential expression in tumors. The small arrow shows positive staining. The large arrow shows negative staining. Figure 3B shows that in poorly differentiated and invasive prostate carcinoma, single cells invading stroma display intense staining (arrow).

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Figures 4A, 4B and 4C show serum stimulated migration of control transfected and T $\beta$ 15 transfected Dunning R- 3327 variants and their growth rate. Figure 4A. Vector control transfected (○, ▽) and T $\beta$ 15 antisense (●, ▽) transfected AT3.1 cell clones. Figure 4B. Vector control transfected (○, ▽) and T $\beta$ 15 sense transfected (●, ▽) AT2.1 cell clones. Data are expressed as the mean  $\pm$  SE (n = 4). Figure 4C. Growth curves of control transfected and T $\beta$ 15 (sense or antisense) transfected Dunning R-3327 clones. Cells from vector control transfected AT2.1 (○), T $\beta$ 15 sense transfected AT2.1 (●), vector control transfected AT3.1 (▽) and T $\beta$ 15 antisense transfected AT3.1 (▽) were plated at initial 10<sup>4</sup> cells/well in RPMI 1640 with 10% FBS and 250 nM dexamethasone in 12-well plates. Cells were harvested and counted at indicated times. Points represent the mean  $\pm$  SE (n = 3).

Figures 5A, 5B, 5C and 5D show immunohistochemical staining of human prostatic carcinoma tissues with an affinity purified polyclonal antibody to thymosin  $\beta$ 15. A. Nonmalignant prostatic epithelia (large arrow) and high-grade prostatic intraepithelial neoplasia (PIN) (small arrow). B. Moderately differentiated prostatic carcinoma showing heterogeneous immunostaining (small arrow, positive; large arrow, negative). C. Poorly differentiated prostatic carcinoma. D. Single cells invading stroma showing intense staining.

25

### Detailed Description of the Invention

Although members of the thymosin  $\beta$  family have been shown to bind and sequester G-actin, they have not previously been demonstrated to alter cell motility. Our studies, however, reveal that

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thymosin  $\beta$ 15 directly regulates cell motility in prostatic carcinoma cells. We have shown that expression of thymosin  $\beta$ 15 is upregulated in highly metastatic prostate cancer cell lines relative to poorly metastatic or nonmetastatic lines. In addition, thymosin  $\beta$ 15 was  
5 expressed in human prostate carcinoma specimens but not in normal human prostate. Although not wishing to be bound by theory, this indicates that  $\beta$ 15 plays a role in the process of metastatic transformation.

10 Accordingly, the evaluation and comparison of levels of transcript (mRNA) or gene product, either normal or mutated, in non-testicular tissue can be both diagnostic and prognostic of particular cancer. For example, an elevated level is indicative of a greater tendency for metastatic activity. Further, by monitoring a particular  
15 neoplastic growth over a period of time and comparing changes in level one can evaluate changes in metastatic activity. The level of  $\beta$ 15 can also be used to determine the severity of a particular tumor. We have found that  $\beta$ 15 expression correlates with gradations of tumor severity. For example, the expression of  $\beta$ 15 in prostate cells is well correlated  
20 with the Gleason grade of prostate carcinoma.

The present invention provides a method of diagnosing cancer, especially prostate, lung, melanoma and breast cancer, in a patient by measuring levels of thymosin  $\beta$ 15 in a biological specimen obtained  
25 from the patient. Levels of thymosin  $\beta$ 15 in the sample greater than a base line level is indicative of cancer. Base line levels can readily be determined by measuring levels of thymosin  $\beta$ 15 in sample of disease free individuals.

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Biological specimens include, for example, blood, tissue, serum, stool, urine, sputum, cerebrospinal fluid and supernatant from cell lysate. Preferably, one uses tissue specimens. The determination of base lines and comparison levels is by standard modes of analysis  
5 based upon the present disclosure.

The present invention also provides a method of prognosis in an individual having cancer, especially prostate, lung, melanoma and breast cancer, by measuring levels of thymosin  $\beta$ 15 in a tumor sample  
10 obtained from a patient to be tested. Expression of thymosin  $\beta$ 15 in said tumor sample greater than a base line level for that particular tissue indicates a higher risk of tumor metastasis. This information can be used by the physician in determining the most effective course of treatment.

15

Changes in a patients condition can be monitored using the methods of the present invention by comparing changes in thymosin  $\beta$ 15 expression levels in the tumor in that subject over time.

20

The present invention further provides a method for determining the metastatic potential of a tumor by measuring the level of thymosin  $\beta$ 15 expression in the tumor. Expression of thymosin  $\beta$ 15 in said tumor greater than a base line level for that particular tissue indicates an increased metastatic potential.

25

Standard detection techniques well known in the art for detecting RNA, DNA, proteins and peptides can readily be applied to detect thymosin  $\beta$ 15 or its transcript to diagnose cancer, especially metastatic cancer or to confirm that a primary tumor has, or has not,

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reached a particular metastatic phase.

Such techniques may include detection with nucleotide probes or  
5 may comprise detection of the protein by, for example, antibodies or  
their equivalent. Preferably, the nucleotide probes may be any that will  
hybridize more strongly to the sequence shown in SEQ ID NO: 1 than  
to other naturally occurring thymosin sequences. Types of probe  
include cDNA, riboprobes, synthetic oligonucleotides and genomic  
10 probes. The type of probe used will generally be dictated by the  
particular situation, such as riboprobes for in situ hybridization, and  
cDNA for Northern blotting, for example. The most preferred probes are  
those which correspond to the DNA of SEQ ID NO: 1. Preferably the  
probe is directed to the thymosin  $\beta$ 15 coding region, i.e., nucleotides  
15 98-232 of SEQ ID NO: 1. Most preferably, the probe is directed to  
nucleotide regions unique to thymosin  $\beta$ 15, e.g., nucleotides 113-133,  
158-169 or 200-232 of SEQ ID NO: 1. Detection of the thymosin  $\beta$ 15  
encoding gene, per se, will be useful in screening for mutations  
associated with enhanced expression. Other forms of assays to detect  
20 targets more readily associated with levels of expression--transcripts  
and other expression products will generally be useful as well. The  
probes may be as short as is required to differentially recognize  
thymosin  $\beta$ 15 mRNA transcripts, and may be as short as, for example,  
15 bases.

25

A probe may also be reverse-engineered by one skilled in the art  
from the amino acid sequence of SEQ ID NO:2. However use of such  
probes may be limited, as it will be appreciated that any one given  
reverse-engineered sequence will not necessarily hybridize well, or at all

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with any given complementary sequence reverse-engineered from the same peptide, owing to the degeneracy of the genetic code. This is a factor common in the calculations of those skilled in the art, and the degeneracy of any given sequence is frequently so broad as to yield a large number of probes for any one sequence.

The form of labelling of the probes may be any that is appropriate, such as the use of radioisotopes, for example,  $^{32}\text{P}$  and  $^{35}\text{S}$ . Labelling with radioisotopes may be achieved, whether the probe is synthesized chemically or biologically, by the use of suitably labelled bases. Other forms of labelling may include enzyme or antibody labelling such as is characteristic of ELISA.

Detection of RNA transcripts may be achieved by Northern blotting, for example, wherein a preparation of RNA is run on a denaturing agarose gel, and transferred to a suitable support, such as activated cellulose, nitrocellulose or glass or nylon membranes. Radiolabelled cDNA or RNA is then hybridized to the preparation, washed and analyzed by autoradiography.

20

In situ hybridization visualization may also be employed, wherein a radioactively labelled antisense cRNA probe is hybridized with a thin section of a biopsy sample, washed, cleaved with RNase and exposed to a sensitive emulsion for autoradiography. The samples may be stained with haematoxylin to demonstrate the histological composition of the sample, and dark field imaging with a suitable light filter shows up the developed emulsion. Non-radioactive labels such as digoxigenin may also be used.

25

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Immunohistochemistry may be used to detect expression of human thymosin  $\beta$ 15 in a biopsy sample. A suitable antibody is brought into contact with, for example, a thin layer of cells, washed, and then contacted with a second, labelled antibody. Labelling may be by  
5 enzyme, such as peroxidase, avidin or by radiolabelling. Chromogenic labels are generally preferable, as they can be detected under a microscope.

More generally preferred is to detect the protein by  
10 immunoassay, for example by ELISA or RIA, which can be extremely rapid. Thus, it is generally preferred to use antibodies, or antibody equivalents, to detect thymosin  $\beta$ 15.

It may not be necessary to label the substrate, provided that the  
15 product of the enzymatic process is detectable and characteristic in its own right (such as hydrogen peroxide for example). However, if it is necessary to label the substrate, then this may also comprise enzyme labelling, labelling with radioisotopes, antibody labelling, fluorescent marker labelling or any other suitable form which will be readily  
20 apparent to those skilled in the art.

Antibodies may be prepared as described below, and used in any suitable manner to detect expression of thymosin  $\beta$ 15.

Antibody-based techniques include ELISA (enzyme linked  
25 immunosorbent assay) and RIA (radioimmunoassay). Any conventional procedures may be employed for such immunoassays. The procedures may suitably be conducted such that: a thymosin  $\beta$ 15 standard is labelled with a radioisotope such as  $^{125}\text{I}$  or  $^{35}\text{S}$ , or an assayable enzyme, such as horseradish peroxidase or alkaline phosphatase and, together

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with the unlabelled sample, is brought into contact with the corresponding antibody, whereon a second antibody is used to bind the first and radioactivity or the immobilized enzyme assayed (competitive assay); alternatively, thymosin  $\beta$ 15 in the sample is allowed to react  
5 with the corresponding immobilized antibody, radioisotope- or enzyme-labelled anti-thymosin  $\beta$ 15 antibody is allowed to react with the system and radioactivity or the enzyme assayed (ELISA-sandwich assay). Other conventional methods may also be employed as suitable.

The above techniques may be conducted essentially as a "one-step"  
10 or "two-step" assay. The "one-step" assay involves contacting antigen with immobilized antibody and, without washing, contacting the mixture with labeled antibody. The "two-step" assay involves washing before contacting the mixture with labeled antibody. Other conventional methods may also be employed as suitable.

15

Enzymatic and radio-labelling of thymosin  $\beta$ 15 and/or the antibodies may be effected by conventional means. Such means will generally include covalent linking of the enzyme to the antigen or the antibody in question, such as by glutaraldehyde, specifically so as not  
20 to adversely affect the activity of the enzyme, by which is meant that the enzyme must still be capable of interacting with its substrate, although it is not necessary for all of the enzyme to be active, provided that enough remains active to permit the assay to be effected. Indeed, some techniques for binding enzyme are non-specific (such as using  
25 formaldehyde), and will only yield a proportion of active enzyme.

It is usually desirable to immobilize one component of the assay system on a support, thereby allowing other components of the system to be brought into contact with the component and readily removed



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without laborious and time-consuming labor. It is possible for a second phase to be immobilized away from the first, but one phase is usually sufficient.

5           It is possible to immobilize the enzyme itself on a support, but if solid-phase enzyme is required, then this is generally best achieved by binding to antibody and affixing the antibody to a support, models and systems for which are well-known in the art. Simple polyethylene may provide a suitable support.

10

          Enzymes employable for labelling are not particularly limited, but may be selected from the members of the oxidase group, for example. These catalyze production of hydrogen peroxide by reaction with their substrates, and glucose oxidase is often used for its good stability,  
15       ease of availability and cheapness, as well as the ready availability of its substrate (glucose). Activity of the oxidase may be assayed by measuring the concentration of hydrogen peroxide formed after reaction of the enzyme-labelled antibody with the substrate under controlled conditions well-known in the art.

20

          Other techniques may be used to detect thymosin  $\beta$ 15 according to preference. One such technique is Western blotting (Towbin et al., *Proc. Nat. Acad. Sci.* 76:4350 (1979)), wherein a suitably treated sample is run on an SDS-PAGE gel before being transferred to a solid  
25       support, such as a nitrocellulose filter. Anti-thymosin  $\beta$ 15 antibodies (unlabelled) are then brought into contact with the support and assayed by a secondary immunological reagent, such as labelled protein A or anti-immunoglobulin (suitable labels including  $^{125}\text{I}$ , horseradish peroxidase and alkaline phosphatase).

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Samples for diagnostic purposes may be obtained from any number of sources. A sample obtained direct from the tumor, such as the stroma or cytosol, may be used to determine the metastatic potential of the tumor. It may also be appropriate to obtain the sample from other biological specimens, such as blood or urine. Such diagnosis may be of particular importance in monitoring progress of a patient, such as after surgery to remove a tumor. If a reference reading is taken after the operation, then another taken at regular intervals, any rise could be indicative of a relapse, or possibly a metastasis. Preferably, the sample is from the tumor itself.

The antibodies may be raised against either a peptide of thymosin  $\beta$ 15 or the whole molecule. Such a peptide may be presented together with a carrier protein, such as an KLH, to an animal system or, if it is long enough, say 25 amino acid residues, without a carrier. Preferred peptides include regions unique to thymosin  $\beta$ 15, such as amino acid 7 to 12 of SEQ ID NO: 2, amino acid 21 to 24 of SEQ ID NO: 2 and amino acid 36 to 45 of SEQ ID NO: 2.

Polyclonal antibodies generated by the above technique may be used direct, or suitable antibody producing cells may be isolated from the animal and used to form a hybridoma by known means (Kohler and Milstein, *Nature* 256:795. (1975)). Selection of an appropriate hybridoma will also be apparent to those skilled in the art, and the resulting antibody may be used in a suitable assay to identify thymosin  $\beta$ 15.

This invention provides a convenient kit for measuring human

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thymosin  $\beta$ 15 levels. This kit includes antibodies or antibody fragments which selectively bind human thymosin  $\beta$ 15 or a set of DNA oligonucleotide primers that allow synthesis of cDNA encoding human thymosin  $\beta$ 15. Preferably, the primers comprise at least 10 nucleotides and hybridizes under stringent conditions to a DNA fragment having the nucleotide sequence set forth in SEQ ID NO:1. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences.

10

DNA encoding human thymosin  $\beta$ 15 and recombinant human thymosin  $\beta$ 15 may be produced according to the methods set forth in copending application U.S.S.N. 08/664,856 the disclosure of which is incorporated herein.

15

All references cited above or below are herein incorporated by reference.

The following Examples serve to illustrate the present invention, and are not intended to limit the invention in any manner.

20

## EXAMPLES

### METHODS

#### RT-PCR Analysis

25

Total RNA from each cell line was digested with RNase free DNase I (GIBCO BRL, Gaithersburg, MD). DNase I digested 5  $\mu$ g of total RNA was reverse transcribed using cDNA Cyling Kit (Invitrogen). The reverse transcription mixture was purified with a Spin Column 300 (Pharmocia, Piscataway, NJ). 10  $\mu$ l of purified cDNA was amplified

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with primer sets of T $\beta$ 15 forward primer:

5'-TATCAGCTAGTGGCTGCACCCGCG-3' (SEQ ID NO:3) and reverse primer: 5'-AAATGCTGACCTTTTCAGTCAGGGT-3' (SEQ ID NO:4); T $\beta$ 4

forward primer: 5'-ACTCTCAATTCCACCA TCTCCCAC-3' (SEQ ID NO:5), reverse primer: 5'-GCCTCTGAGCAGATCGTCTCTCCTTG-3' (SEQ ID NO:6); and T $\beta$ 10 forward primer:

5'-ATAATATCCCTGGGCAAACCGGTG-3' (SEQ ID NO:7), reverse primer: 5'-GAGTGGAG TACCTGGAGCGCGAGC-3' (SEQ ID NO:8),

respectively. PCR amplification was performed in 50  $\mu$ l of PCR reaction buffer (50 mM KCl, 10 mM Tris [pH 8.5], 1.5 mM MgCl<sub>2</sub>) with 1 mM of dNTPs, 50 pmol of each primer, and 2.5 U of Taq polymerase (GIBCO BRL), overlaid with 50  $\mu$ l of mineral oil (Sigma). The PCR profile was 94°C, 30 sec; 60°C, 30 sec; and 72°C, 2 min for 30 cycles.

Control studies of the RT-PCR were conducted using aliquats from the same samples and amplified with primers to the  $\beta$ -actin gene (Clontech, Palo Alto, CA). Amplification products were separated on 1.4% agarose gels.

#### In situ hybridization

Antisense and sense T $\beta$ 15 mRNA probes were prepared using T $\beta$ 15 cDNA inserted into the eukaryotic expression vector pcDNA3 (Invitrogen) as template and a digoxigenin RNA labeling kit (Boehringer Mannheim). Formalin-fixed paraffin-embedded sections were dewaxed, rehydrated, and digested with proteinase K (50  $\mu$ g/ml) in 100 mM Tris, 50 mM EDTA buffer (pH 8) for 8 min at 37°C. Hybridization was performed in an automated instrument (Ventana Medical Systems, Tuscon, AZ) for 60 min at 42°C with 10 pM digoxigenin-labeled riboprobe in 100  $\mu$ l of hybridization buffer (50% deionized formamide, 4x SSC, 10% dextran sulfate, 1% SDS, and denatured herring sperm

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DNA (400 µg/ml)) per section under a liquid cover slip. The highest stringency of posthybridization washes was at 45°C for 15 min in 0.1 x SSC. Bound digoxigenin-labeled probe was detected by anti-digoxigenin alkaline phosphatase conjugate and visualized by nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) color reaction. Sections were counterstained with nuclear fast red.

#### Cell motility

Migration of transfectants was studied using a multiwell chamber assay as previously described (Kunda, et al., *J. Cell Biol.* 130, 725 (1995)) 48-well chemotaxis chambers were overlaid with 8-µm porosity polycarbonate filters (Nucleopore Corp., Pleasanton, CA) precoated with PBS containing 11.5 µg/ml fibronectin (Cappel Organon Technica, Durham, NC). The migration of 5,000 cells placed in the upper well toward fetal bovine serum in the lower well was assayed following a 4 hour incubation at 37°C. After removal of cells from the upper side of the filters, cells that had passed through the filters and adhered to the lower side were fixed in formalin, washed with PBS and stained with Gill's triple strength hematoxylin (Polysciences, Warrington, PA) and counted under light microscopy.

#### Immunohistochemical staining

Human prostate cancer sections were studied using an immunoperoxidase ABC kit (Vector, Burlingame, CA). Briefly, the 5 µm tissue sections were deparaffinized in xylene, rehydrated in graded alcohols, and blocked for endogenous peroxidase by 3% hydrogen peroxide (Sigma) in methanol for 30 min. The sections were treated

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with normal goat serum for 30 min and then incubated with an affinity purified anti T $\beta$ 15 C-terminal peptide antibody for 2 h at room temperature at 1:100 (v/v) dilution, followed by incubation with a biotinylated goat anti-rabbit IgG antibody for 30 min. After incubation  
5 with a preformed ABC complex for 30 min, specifically bound antibodies were visualized by using peroxidase substrate, 3, 3'-diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with Gill's hematoxylin.

## 10 RESULTS

### Cloning of Human Thymosin $\beta$ 15 by RT-PCR

DNase I digested 5  $\mu$ g of total RNA from human prostatic carcinoma cell line PC-3 was reverse transcribed using cDNA Cycling Kit (Invitrogen). The reverse transcription mixture was purified with a  
15 Spin Column 300 (Pharmacia, Piscataway, NY). 10  $\mu$ l of purified cDNA reaction was amplified with primers F1 (5'-TATCAGCTAGTGGCTGCACCCGCG-3') (SEQ ID NO:8) and R1 (5'-AAATGCT GACCTTTCAGTCAGGGT-3') (SEQ ID NO:9) designed to anneal to the outer ends of the thymosin  $\beta$ 15 sequence. PCR  
20 amplification was performed in 50  $\mu$ l of PCR reaction buffer (50 mM KCl, 10 mM Tris [pH 8.5], 1.5 mM MgCl<sub>2</sub>) with 1 mM of dNTPs, 50 pmol of each primer, and 2.5 U of Taq polymerase (GIBCO BRL), overlaid with 50  $\mu$ l of mineral oil (Sigma). The PCR profile was 94°C, 30 sec; 60°C, 30 sec; and 72°C, 2 min for 30 cycles. Control studies  
25 of the RT-PCR were conducted using aliquats from the same samples and amplified with primers to the  $\beta$ -actin gene (Clontech, Palo Alto, CA). Amplification products were separated on 1.6% agarose gels. The amplified PCR product was ligated to pCR using TA cloning kit (Invitrogen, San Diego, (CA), and then DNA sequenced. The sequence

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of the PCR product of human prostatic carcinoma cells amplified by the thymosin  $\beta$ 15 primers is set forth in Figure 1 (SEQ ID NOS: 1 and 2).

#### **Expression of T $\beta$ 15 mRNA in human prostate cancer**

5           To determine whether this thymosin family member may be expressed in human prostate cancer, we examined human prostatic carcinoma cell line PC-3 by RT-PCR with forward and reverse primers for thymosin  $\beta$ 15. The PC-3 cells showed a low level of thymosin  $\beta$ 15 expression. The DNA sequence of the amplified PCR product was  
10   100% identical to the rat thymosin  $\beta$ 15 sequence. We conducted in situ hybridization study on samples from patients with varying grades of prostatic carcinomas using a thymosin  $\beta$ 15 probe. The tissue sections allowed direct comparison of normal and malignant elements on the same samples. The stromal elements within and around the  
15   tumor cell masses, as well as the nonmalignant prostatic epithelium adjacent to the tumor showed little background hybridization with the thymosin  $\beta$ 15 antisense probe. In contrast, specific tumor cell islands exhibited a strong specific thymosin  $\beta$ 15 signal when probed with antisense (Figure 3A, small arrow) but not with a sense RNA probe  
20   (data not shown). Although nearly all of the tumor cells in the positive islands expressed thymosin  $\beta$ 15 mRNA, not all patient specimens were positive and not all islands in a single prostate were positive (Figure 3A, large arrow). The majority of the negative tumor cells were in non-invasive in situ carcinomas whereas highly invasive tumors were  
25   consistently positive (Figure 3B). Thus a novel  $\beta$  thymosin, first detected in metastatic rat prostate carcinoma cell lines, is upregulated in human prostate cancer.

#### **Effect of T $\beta$ 15 on cell motility**

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To determine whether thymosin  $\beta$ 15 expression had an effect on cell motility, we transfected highly motile AT3.1 cells with a eukaryotic expression vector (pcDNA3) containing the thymosin  $\beta$ 15 gene in antisense orientation driven by the constitutive human cytomegalovirus promoter. The transfected cells growing in selective (G418) media were examined for expression of antisense transcripts of the thymosin  $\beta$ 15 gene by strand-specific polymerase chain reaction (PCR) amplification (Zhou, et al., *Cancer Res.* 52, 4280-4285 (1992). Analysis of cell motility in a multiwell Boyden chamber apparatus (Boyden, S. V., *J. Exp. Med.* 115, 453-466 (1962)) using fetal bovine serum as a migration stimulus revealed that the motility of the transfectants which showed expression of antisense transcripts was significantly reduced relative to the vector-only controls (Figure 4A). Two antisense transfected clones which did not express antisense transcripts failed to show any decreased rate of cell motility (data not shown). In a further experiment, poorly motile AT2.1 cells, transfected with sense thymosin  $\beta$ 15 constructs and confirmed to express thymosin  $\beta$ 15 by Northern analysis, were shown to have significantly increased stimulated motility relative to their vector controls (Figure 4B). Both the sense and antisense thymosin  $\beta$ 15 transfectants showed similar rates of cell proliferation relative to controls suggesting differential specificity for different cellular events (Figure 4C). The results demonstrate that thymosin  $\beta$ 15, which is upregulated in the highly motile AT3.1 and AT6.1 Dunning tumor cell lines, is a positive regulator of cell motility which is an important component of cancer metastasis.

#### **Immunohistochemical detection of T $\beta$ 15 in prostate carcinoma**

A polyclonal antibody was raised against a peptide representing



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the 11 C-terminal amino acids of thymosin  $\beta$ 15. Synthesized peptide was coupled with a carrier, keyhole limpet hemocyanin (KLH), and injected into rabbits. Antiserum was affinity-purified over the C-terminal peptide coupled CNBr-activated sepharose 4B column. To test the  
5 specificity of the purified antibody, we performed Western analysis of the GST/thymosin  $\beta$  fusion proteins with the affinity-purified anti C-terminal antibody. The purified antibody strongly reacted with GST-thymosin  $\beta$ 15 fusion protein, but did not cross react with GST-thymosin  $\beta$ 4, and not with GST alone showing its specificity.

10

We used the affinity purified polyclonal thymosin  $\beta$ 15 antibody for immunohistochemical study of human prostate carcinoma. The results are summarized below in Table 1. The thymosin  $\beta$ 15 immunostaining was observed in the cytoplasm of epithelial cells in  
15 neoplastic prostates but not in normal prostates and not in the stromal cells (Figure 5A, large arrow). Among the investigated malignant epithelia, the poorly differentiated prostate carcinomas displayed the most extensive and intense thymosin  $\beta$ 15 immunoreaction (Figure 5C), followed by moderately differentiated prostate carcinomas in which not  
20 all carcinomas expressed thymosin  $\beta$ 15 showing partial positivity (Figure 5B). In some cases, high-grade prostatic intraepithelial neoplasia (PIN) showed thymosin  $\beta$ 15 immunostaining, but to a lesser extent (Figure 5A, small arrow). In poorly differentiated invasive carcinoma, single cells invading stroma displayed intense staining (Figure 5D). The  
25 expression of thymosin  $\beta$ 15 well correlated with Gleason grade of prostate carcinoma.

TABLE 1

THYMOSIN  $\beta$ 15 EXPRESSION IN HUMAN PROSTATE CARCINOMA

- 24 -

| Prostate          | No. | Negative <sup>a</sup> | Partial <sup>b</sup> | Positive <sup>c</sup> |
|-------------------|-----|-----------------------|----------------------|-----------------------|
| BPH               | 2   | 2                     | 0                    | 0                     |
| Ca Gleason 2 ~ 5  | 5   | 3                     | 2                    | 0                     |
| Ca Gleason 6 ~ 8  | 25  | 4                     | 7                    | 14                    |
| Ca Gleason 9 ~ 10 | 6   | 0                     | 1                    | 5                     |
| Ca (with met)     | 3   | 0                     | 1                    | 2                     |

(BPH - Benign Prostate Hyperplasia; CA-Carcinoma)

- 5      a.    less than 10% cells showing positivity  
        b.    heterogeneous staining with 30 ~ 75% of cells showing positivity  
        c.    homogeneous staining with 75 ~ 100% of cells showing positivity

10

This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

15

- 25 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT: BRUCE R. ZETTER AND LERE BAO
- (ii) TITLE OF THE INVENTION: A METHOD FOR DIAGNOSIS AND PROGNOSIS OF  
CANCER
- (iii) NUMBER OF SEQUENCES: 8
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: DIKE, BRONSTEIN, ROBERTS & CUSHMAN
  - (B) STREET: 130 WATER STREET
  - (C) CITY: BOSTON
  - (D) STATE: MA
  - (E) COUNTRY: USA
  - (F) ZIP: 02019
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette
  - (B) COMPUTER: IBM Compatible
  - (C) OPERATING SYSTEM: DOS
  - (D) SOFTWARE: FastSEQ Version 1.5
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE: 17 JUN 1996
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: DAVID, RESNICK S
  - (B) REGISTRATION NUMBER: 34,235
  - (C) REFERENCE/DOCKET NUMBER: 46403
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 617-523-3400
  - (B) TELEFAX: 617-523-6440
  - (C) TELEX: 200291 STRE

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 412 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTISENSE: NO
- (v) FRAGMENT TYPE:
- (vi) ORIGINAL SOURCE:
- (ix) FEATURE:
  - (A) NAME/KEY: Coding Sequence

- 26 -

(B) LOCATION: 98...232  
 (D) OTHER INFORMATION: Exon 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

|  |     |
|--|-----|
| TATCAGCTAG TGGCTGCACC CGCGAACACC ACCCTGGTCC GGAGTAGCTG CGGACAGAAT  | 60  |
| TGCTGGCCTA GTAGAAGCTT TGGAACGAGC AGTCAAG ATG AGT GAT AAA CCA GAC   | 115 |
| Met Ser Asp Lys Pro Asp  |     |
| 1 5  |     |
| TTA TCA GAA GTT GAA ACA TTT GAC AAA TCA AAG TTG AAG AAG ACT AAT    | 163 |
| Leu Ser Glu Val Glu Thr Phe Asp Lys Ser Lys Leu Lys Lys Thr Asn    |     |
| 10 15 20   |     |
| ACT GAA GAA AAG AAT ACT CTT CCT TCG AAG GAA ACT ATC CAG CAG GAG    | 211 |
| Thr Glu Glu Lys Asn Thr Leu Pro Ser Lys Glu Thr Ile Gln Gln Glu    |     |
| 25 30 35   |     |
| AAA GAA TAT AAT CAA AGA TC ATAAAATGAG ATTCTCCTCT CAAGAGCAAC TTCAAC | 267 |
| Lys Glu Tyr Asn Gln Arg Ser  |     |
| 40 45  |     |
| TTTGCTGGAT AGTCTTGGAT TTAGACATGT TTCTGTAAAC CTATCCAATA TGTAGACATT  | 327 |
| TTAGGCGGTT CCTGATAGGT TCTTAAGTAC CCTGACTGAA AGGTCAGCAT TTAACACCAA  | 387 |
| TCATTAAATG TGTTTCCAC TGCTC   | 412 |

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

|   |  |
|---|--|
| Met Ser Asp Lys Pro Asp Leu Ser Glu Val Glu Thr Phe Asp Lys Ser |  |
| 1 5 10 15   |  |
| Lys Leu Lys Lys Thr Asn Thr Glu Glu Lys Asn Thr Leu Pro Ser Lys |  |
| 20 25 30  |  |
| Glu Thr Ile Gln Gln Glu Lys Glu Tyr Asn Gln Arg Ser             |  |
| 35 40 45  |  |

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

- 27 -

(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TATCAGCTAG TGGCTGCACC CGCG

24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAATGCTGAC CTTTCAGTCA GGGT

24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACTCTCAATT CCACCATCTC CCAC

24

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA  
(iii) HYPOTHETICAL: NO  
(iv) ANTISENSE: NO  
(v) FRAGMENT TYPE:  
(vi) ORIGINAL SOURCE:

- 28 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCCTCTGAGC AGATCGTCTC TCCTTG

26

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATAATATCCC TGGGCAAACC GGTG

24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGTGGAGTA CCTGGAGCGC GAGC

24

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What is claimed is:

1. A method of diagnosing cancer in a patient, comprising: obtaining a biological specimen; measuring levels of thymosin  $\beta$ 15 in said specimen, wherein levels of thymosin  $\beta$ 15 in said sample greater than a base line level is indicative of cancer.
2. The method of claim 1, wherein the biological specimen is selected from blood, tissue, serum, stool, urine, sputum, cerebrospinal fluid and supernatant from cell lysate.
3. A method of prognosis in an individual having cancer comprising, obtaining a tumor sample from a patient to be tested and measuring levels of thymosin  $\beta$ 15 in said tumor sample, wherein levels of thymosin  $\beta$ 15 in said tumor sample greater than a base line level indicates a higher risk of tumor metastasis.
4. The method of claim 1 or 3, wherein the cancer is prostate, lung, melanoma, breast.
5. A method for determining the metastatic potential of a tumor comprising measuring the level of thymosin  $\beta$ 15 expression in said tumor, wherein levels of thymosin  $\beta$ 15 in said tumor greater than a base line level indicates an increased metastatic potential.
6. The method of claim 1, 3 or 5 wherein the level of mRNA expressing thymosin  $\beta$ 15 is measured.
7. The method of claim 6, wherein the mRNA is detected by use of an RNA dependent polymerase chain reaction.

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8. The method of claim 1, 3 or 5 wherein the level of the thymosin  $\beta$ 15 protein is measured.

9. The method of claim 4, wherein the mRNA is detected by Northern blot analysis by hybridizing mRNA from said biological specimen to a thymosin  $\beta$ 15 nucleotide probe.

10. A method for measuring human thymosin  $\beta$ 15 levels which comprises the steps of:

a. contacting a sample or preparation thereof with an antibody or antibody fragment which selectively binds human thymosin  $\beta$ 15, and

b. detecting whether said antibody or said antibody fragment is bound by said sample and thereby measuring the levels of human thymosin  $\beta$ 15 present, wherein the human thymosin  $\beta$ 15 level is prognostic of an individuals condition, wherein a negative result is indicative of a better prognosis than a positive result.

11. The method according to claim 10, wherein said antibody, or said antibody fragment, is detectably labelled.

12. A kit for measuring human thymosin  $\beta$ 15 levels comprising, a set of DNA oligonucleotide primers, said set allowing synthesis of cDNA encoding human thymosin  $\beta$ 15.

13. The kit of claim 12, wherein said primers comprise at least 10 nucleotides and hybridizes under stringent conditions to a DNA fragment having the nucleotide sequence set forth in SEQ ID NO:1.



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14. A kit for measuring human thymosin  $\beta$ 15 levels comprising an antibody or antibody fragment which selectively binds human thymosin  $\beta$ 15.

| Species      | MSDKPDIAE  | TEKFDKSKLE | KTETIQEKNPL  | PSKETIEQEK  | QAGES  |
|--------------|------------|------------|--------------|-------------|--------|
| Ratthymb4    | MSDKPDIAE  | TEKFDKSKLE | KTETIQEKNPL  | PSKETIEQEK  | QAGES  |
| Bovthymb9    | QAQDKPDJGE | INSFDKAKLE | KTETIQEKNJIL | PTKETIEQEK  | QAK    |
| Ratthymb10   | MAQDKPDJGE | IASFDKAKLE | KTETIQEKNJIL | PIKETIEQEE  | RSETIS |
| Troutthymb11 | ACSDKPNJLE | VASFDKIKLE | KTETIQEKNPI  | PIKETIEQEE  | QAS    |
| Troutthymb12 | ACSDKPDIAE | VSNFDKIKLE | KTETIQEKNPL  | PIKETIIEQEE | QAT    |
| Humanthymb15 | MSDKPDUSE  | VETFDKSKLE | KTINIEEKNJIL | PSKETIQEKK  | EYNORS |

FIG. 2

FIG. 3A

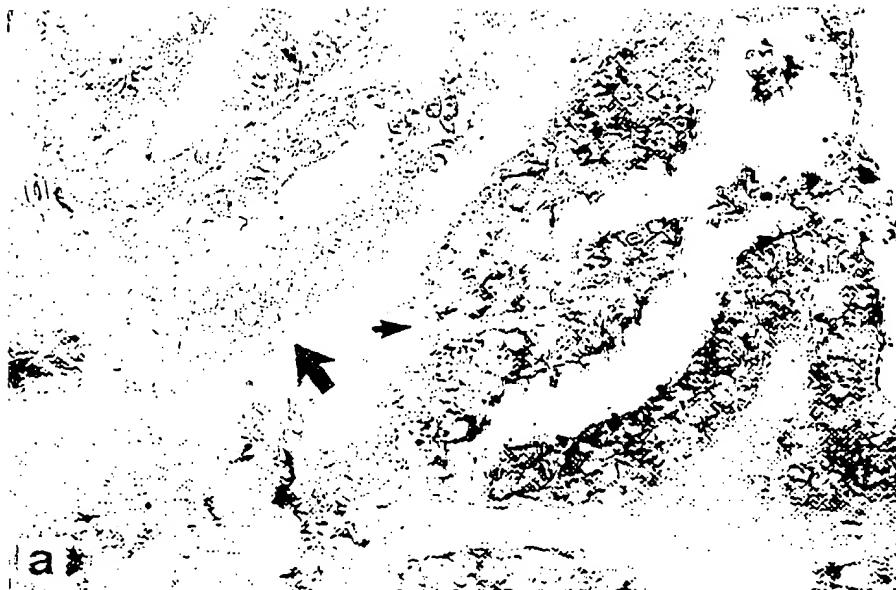
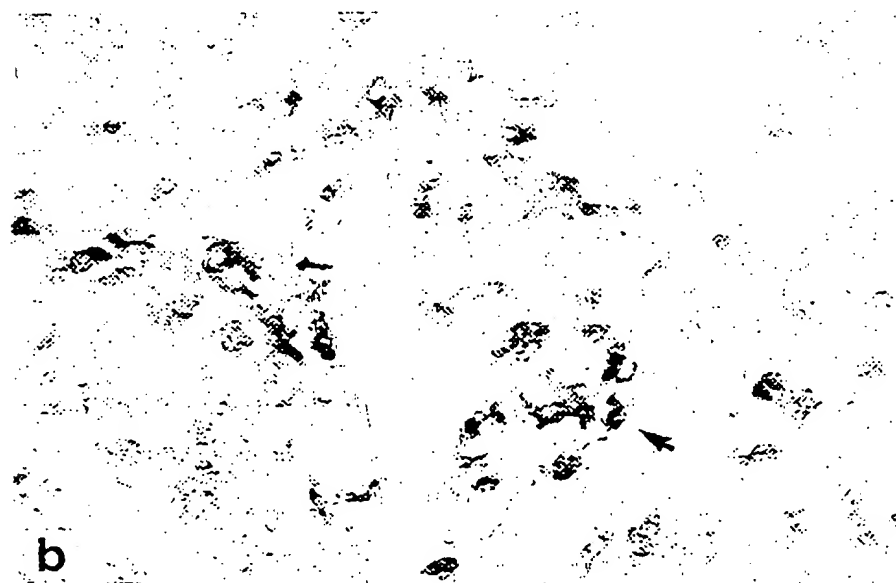


FIG. 3B



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FIG. 4A

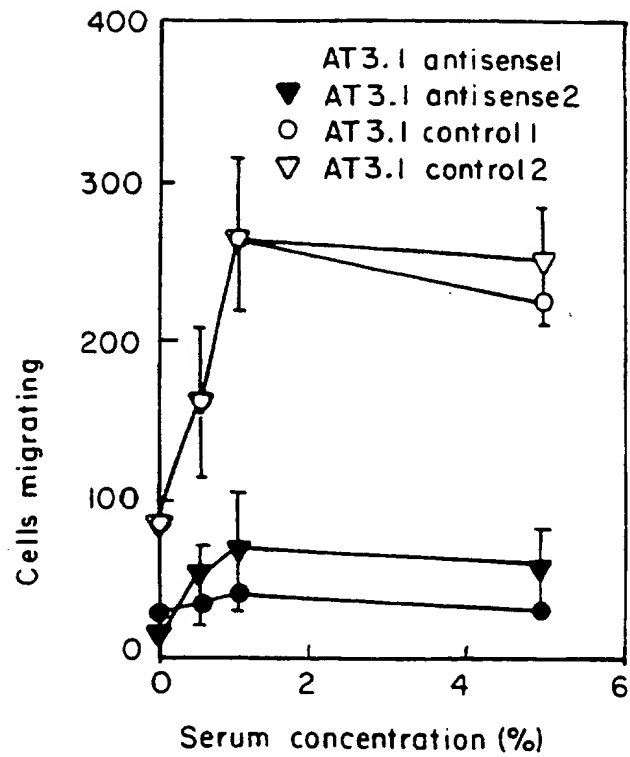
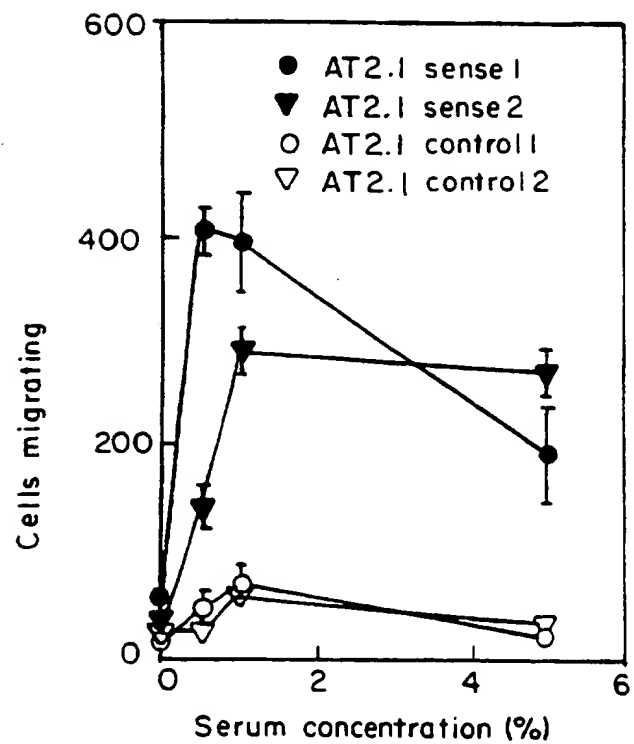


FIG. 4B



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FIG. 4C

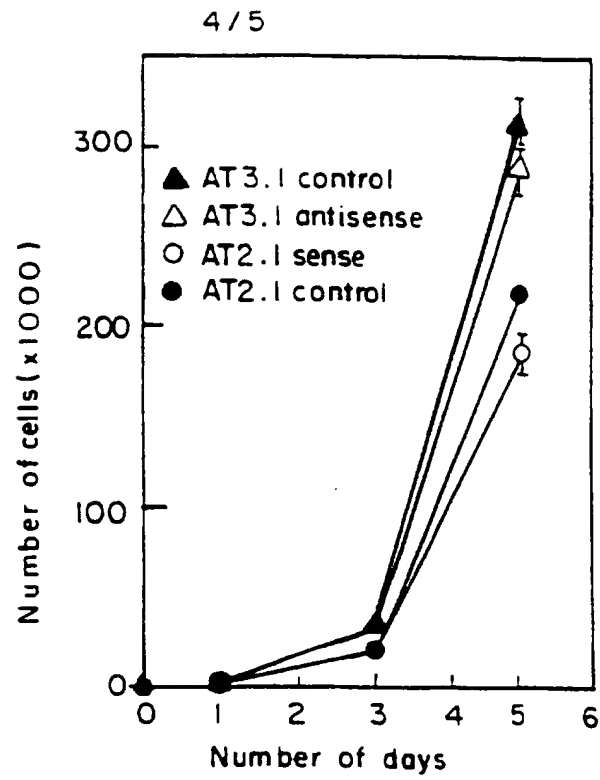




FIG. 5A

FIG. 5B

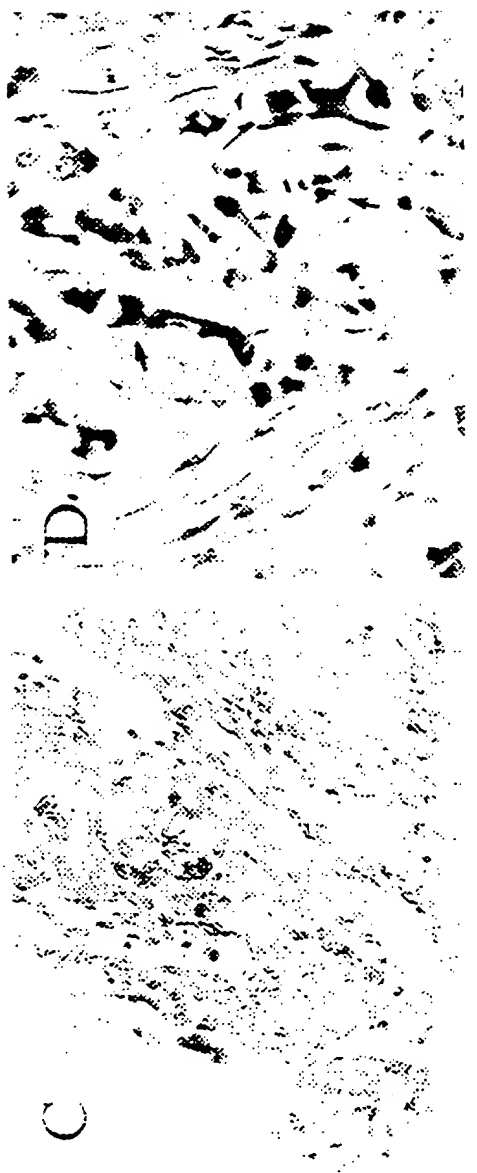


FIG. 5C

FIG. 5D

## INTERNATIONAL SEARCH REPORT

Interns il Application No  
PCT/US 97/10306A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 G01N33/574 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 G01N C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| P,X        | BAO, LERE ET AL: "Thymosin.beta.15: a novel regulator of tumor cell motility upregulated in metastatic prostate cancer" NAT. MED. (N. Y.) (1996), 2(12), 1322-1328 CODEN: NAMEFI;ISSN: 1078-8956, XP002041674 see the whole document<br>--- | 1-14                  |
| P,X        | D.S. COFFEY: "Prostate Cancer metastasis: Talking the walk" NAT. MED. (N. Y.) (1996), 2(12), 1322-1328 CODEN: NAMEFI;ISSN: 1078-8956, XP002042495 see page 1305, right-hand column, line 24 - line 42<br>---<br>-/--                        | 1-16                  |

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

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- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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\*S\* document member of the same patent family

Date of the actual completion of the international search

3 October 1997

Date of mailing of the international search report

17. 10. 97

Name and mailing address of the ISA

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Authorized officer

Hoekstra, S

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/10306

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| A          | WETERMAN M A ET AL: "Thymosin beta-10 expression in melanoma cell lines and melanocytic lesions: a new progression marker for human cutaneous melanoma." INTERNATIONAL JOURNAL OF CANCER, vol. 53, no. 2, 21 January 1993, pages 278-284, XP002041675<br>see the whole document<br>---  | 1-14                  |
| A          | US 5 248 591 A (PUENTE FERNANDO D) 28 September 1993<br>see the whole document<br>---   | 1-16                  |
| A          | BIOLOGICAL ABSTRACTS, vol. 98, Philadelphia, PA, US; abstract no. 165501,<br>BAO L ET AL: "Differential Expression of a Novel mRNA in Tumor cell Lines with Variant Metastatic Potential." XP002041676<br>cited in the application<br>see abstract<br>&<br>EIGHTY-SIXTH ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, TORONTO, ONTARIO, CANADA, MARCH 18-22, 1995. PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING 36 (0). 1995. 85. ISSN: 0197-016X,<br>----- | 1-16                  |



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/10306

Patent document  
cited in search report

Publication  
date

Patent family  
member(s)

Publication  
date

US 5248591 A

28-09-93

NONE